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THE DISTRIBUTION AND LOCALIZATION OF CHOLINESTERASES IN NERVE CELLS

BY

EZIO GIACOBINI

STOCKHOLM 1959

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From the Department of Physiology, Karolinska Institutet, Stockholm.

The Distribution and Localization of Cholinesterases in Nerve Cells.

By

EZIO GIACOBINI.

STOCKHOLM 1959.

Stockholm 1959. Kungl. Boktr. P. A. Norstedt & Söner

This survey is based on the following 7 papers:

I. GIACOBINI, E. Histochemical Demonstration of AChE Activity in Isolated Nerve Cells. Acta Physiol. Scand. 1956. *36*. 276.

II. GIACOBINI, E. and J. ZAJICEK. Quantitative Determination of Acetylcholinesterase in Individual Nerve Cells. Nature, 1956. *177*. 185.

III. GIACOBINI, E. Quantitative Determination of Cholinesterase in Individual Sympathetic Cells. J. Neurochem. 1957. *1*. 234.

IV. GIACOBINI, E. and B. HOLMSTEDT. Cholinesterase Content of Certain Regions of the Spinal Cord as Judged by Histochemical and Cartesian Diver Technique. Acta Physiol. Scand. 1958. *42*. 12.

V. GIACOBINI, E. Quantitative Determination of Cholinesterase in Individual Spinal Ganglion Cells. Acta Physiol. Scand. 1959. *45*.

VI. GIACOBINI, E. The Intracellular Distribution of Cholinesterase in the Nerve Cell Studied by Means of a Quantitative Micromethod. Acta Physiol. Scand. 1957. *42*. Suppl. 145. 49.

VII. GIACOBINI, E. Determination of Cholinesterase in the Cellular Components of Neurones. Acta Physiol. Scand. 1959. *45*.

The above figures will be used in the following text when making references to these papers.

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Introduction.

This survey was developed from a series of investigations performed at the Department of Physiology, Karolinska Institutet, Stockholm, during the years 1954—1958 and deals with the distribution and the localization of ChE in nerve cells studied by means of different methods. The results of the single investigations have been reported in the 7 papers listed on page 3 and quoted in the course of the text with corresponding roman figures. The investigations have been supported by grants from Karolinska Institutet (Reservationsanslaget).

a) *Definition and nomenclature of cholinesterases.*

Although the existence in the blood of an enzyme responsible for the destruction of acetylcholine (ACh) was suggested by DALE as early as 1914, it was not until 1932, that STEDMAN, STEDMAN and EASSON were able to prepare it for the first time. They obtained from horse serum, an enzyme which they considered capable of splitting specifically ACh and which they called "cholinesterase".

Acetylcholinesterase (AChE), (or specific cholinesterase, true cholinesterase, "e" type, cholinesterase, acetocholinesterase, cholinesterase I) is the enzyme which catalyses the hydrolysis of the acetic-acid ester of choline, ACh.

There exist other enzymes called non-specific cholinesterases (non spec. ChE), (or pseudo cholinesterase, "s" type, unspecified cholinesterases, cholinesterase, butyro- and propiono cholinesterase, cholinesterase II) which can hydrolyse other esters in addition to choline esters even if the latter are split more rapidly.

It has however been demonstrated by ADAMS and WHITTAKER (1949) that true or specific cholinesterases are also able to hydrolyse many non-choline esters. In any case it is now accepted that there are at least two groups of esterases, which can be defined by means of their substrate specificity, their sensitivity to inhibitory substances, their characteristic activity-pS curves and the material from which they can be isolated.

In this paper the symbol AChE has been chosen to signify the first type of esterase while the second type will be represented by: non spec. ChE. The symbol ChE (cholinesterase) will be used to represent all enzymes capable of splitting choline esters irrespective of their specificity for certain substrates or inhibitors.

b) Purpose of the investigation.

In connection with the presumed physiological role of ACh in the mechanism of nerve activity a great deal of work has been devoted in the last 20 years to the study of AChE activity of different parts of the nervous system both by means of biochemical methods and by means of histochemical techniques on tissue sections (for ref. see paper I. p. 276).

A central point of these studies has been to map the enzyme distribution throughout the whole nervous system and to localize it specifically into certain structures within the cell.

As previously discussed (see papers: I, p. 276; III p. 234 and VII discussion) it is extremely difficult to interpret in this respect results of chemical analysis of homogenates of gross specimens of tissue where nervous and non-nervous tissue (glia cells, vascular tissues, erythrocytes etc.) are mixed together, and to correlate their activity to that of single cell components.

Histochemical methods are very useful in providing a more exact demonstration of enzyme localization in cells but owing to diffusion of the products of the histochemical reaction, no conclusion can be drawn from the histochemical picture about the localization of the enzyme in the nerve cell; on the other hand histochemical methods are not adapted to quantitative assessment of ChE activity (cf. discussion in paper IV page 23).

For these reasons the determination of ChE was attempted by means of a modified histochemical technique on single neurones and their constituent parts isolated by means of micromanipulation. (See paper I.)

For the quantitative evaluation of ChE activity in single nerve cells and in isolated parts of cells a microgasometrique technique, the cartesian diver of LINDERSTRÖM-LANG (1937), has been employed. (See papers II, III, VII.)

The investigation was performed on spinal, sympathetic and parasympathetic ganglion cells, anterior and lateral horn cells of the spinal cord, muscle spindles and muscle end plates; the

Table I.

Preparation	Substrate	Inhibitor[1]	Types of enzyme activity determined
1	ACh[2]	none	All ChEs
2	ACh	Mipafox (5×10^{-4} M)	AChE
3	ACh	BW 284 c 51 (4.8×10^{-4} M)	non spec. ChE
4	ACh	Mipafox + BW 284 c 51	control
5	BuCh		predom. non spec. ChE
6	BuCh	BW 284 c 51 (4.8×10^{-4} M)	non spec. ChE
7	MeCh		AChE

[1] For definition and properties of the inhibitors see Holmstedt 1957 a.

[2] In a part of the experiments AThCh and BuThCh instead of ACh and BuCh were employed. In such a case the activity values were corrected according to paper VII, material and methods, 5.

animals used in most experiments were the rat and frog. Control experiments were performed with a macrochemical method (electrometric method) on homogenates of rat (see paper IV), and frog brain. (GIACOBINI and HOLMSTEDT unpublished results.)

The distinction between different types of ChE has been done by employing specific substrates (ACh and BuCh, butyrylcholine, and MeCh, acetyl-β-metylcholine) and specific inhibitors. (See Table I.) Thus with the help of these three different techniques, the histochemical, microchemical and macrochemical, the elucidation of the following points has been attempted:

1) demonstration of the presence, quantitative determination and comparison of the AChE activity of single isolated nerve cells from different regions.

2) study of the distribution of the enzyme in different parts of the nerve cell and of its intracellular localization.

For a detailed description of the methods used it is referred to the single papers listed on page 3 and quoted in the course of the text.

PART I.

Distribution of ChE in Nerve Cells and Nerve Structures of Different Origin.

a) Sympathetic ganglion cells.

The cholinesterase activity of the sympathetic ganglions has been the subject of several investigations performed partly by means of macrochemical methods on homogenates of entire ganglions and partly on tissue sections by means of histochemical techniques.

The literature on this subject has been reviewed in III (see page 234) but in general it can be said that both kinds of methods could demonstrate the presence of both ChEs in the ganglions and suggested the synapsis as the specific location of AChE.

This suggestion was mainly based on the marked decrease of AChE activity following the section of the preganglionic fibres (Brücke 1937, Couteaux and Nachmansohn 1940, Sawyer and Hollinshead 1945) and on the fact that in the superior cervical ganglion of the cat, the enzyme could only be demonstrated histochemically in some ganglion cells and it was found mostly in the preganglionic fibres, while no enzyme could be demonstrated in the postganglionic fibres (Koelle 1951—54).

Further theoretical support of a synaptic localization of the enzyme was given of course by the demonstration that ACh is liberated in the eserinized ganglion (Kibjakow, 1933) when the preganglionic nerve trunk is stimulated (Feldberg *et al.* 1934—35) and by the suggestion that this liberation takes place at the level of the synapsis.

An attempt to study the localization of the enzyme in the sympathetic ganglions by means of a modified thiocholine histochemical method performed on single ganglion cells of the rat, frog and cat isolated by means of microdissection gave however some results differing from those previously mentioned (paper I). In the superior cervical ganglion of the rat, on the basis of their AChE activity the cells could be divided into three groups: about 10 % of the total number of cells showed a high AChE activity after an incubation time of about 5 min, another group of cells,

approximately 40 % of the total number, showed a moderate activity after the same incubation time and finally about 40—50 % of the cells showed a very slight activity or no activity at all. Some of these AChE negative cells were incubated for 24 hours without obtaining any crystal formation whatsoever.

An exact assessment of the AChE positive cells and an exact evaluation of the different levels of enzyme activity could not be done by histochemical methods, owing to some unfavourable conditions. An intracellular localization of the enzyme was also difficult to establish, but from the histochemical picture it was possible to suggest a cytoplasmic localization of the enzyme in single cells and in small parts of cytoplasm isolated by micromanipulation (cf. Results, paper I, page 283 and 286). It was also found that the sympathetic ganglions in the frog had the highest number of AChE-bearing cells, followed by the rat and lastly the cat.

Most of the preganglionic fibres entering the ganglions showed a strong AChE activity and also a certain number of the postganglionic fibers were strongly positive.

No morphological or topographical difference could be found between the cells belonging to these three types.

Capsular glial cells showed a strongly positive reactior for non spec. ChE.

The demonstration of these three types of cells (KOELLE 1951—54, GIACOBINI 1956) by means of a histochemical method has been recently confirmed by HOLMSTEDT and SJÖKVIST (1957) employing also a modified thiocholine method (HOLMSTEDT 1957) on sections of sympathetic ganglions of the cat.

These authors found that in the superior cervical ganglion 0.5 % of the cells showed high intensity of staining, 20 % moderate staining, and 80 % no staining at all.

They also found a significant difference between the incidence of these cells in different ganglions, *e. g.* the heavily stained cells were found to be more numerous in the stellate ganglion than in the superior cervical.

The ChE activity of the sympathetic ganglions was finally studied by means of a microquantitative method, the diver technique (paper III), on isolated cells of the rat and frog.

The results of this investigation, reported in paper III (see Table I, II, III) are presented in this work in Table II (rat) together with the values of the activity per unit volume of the single cell bodies and axons.

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Table II.

AChE activity of different cells or part of cells of the nervous system of the rat.[1]

Type of cell or structure	Cell body		Axon	Other structures	
	Total ChE act. $\mu l\ CO_2/\mu^3/h \times 10^{-6}$	ChE act. per unit volume $\mu l\ CO_2/\mu^3/h \times 10^{-6}$	ChE act. per unit volume $\mu l\ CO_2/\mu^3/h \times 10^{-6}$	Total ChE act. $\mu l\ CO_2/\mu^3/h \times 10^{-6}$	ChE act. per unit volume $\mu l\ CO_2/\mu^3/h \times 10^{-6}$
Anterior horn cell	0.71—6 (23) $M_1 = 1.17 \pm 0.92$ $M_2 = 5.0 \pm 0.22$	49—4 100 (23) $M_1 = 202 \pm 40$ $M_2 = 550 \pm 51$	208—3 800 (4) in. part 130—2 340 (10) dist. part		
dendrite					341—2 250 (4) M = 1 124
nucleus				0.002—0.008 (7) M = 0.005	46—81 (7) M = 61
nucleolus				—(3)	—(3)
Symp. ganglion c.	0.11—2.2 (24) M = 0.70	5.1—185 (24) M = 46	26—350 (24) M = 175		
Paras. ganglion c.	1.2—4 (10) M = 2.85	17.2—80 (10) M = 61			
Spinal ganglion c.	0.06—3 (50) $M_1 = 0.27 \pm 0.02$ $M_2 = 1.93 \pm 0.03$	0.71—123 (50) $M_1 = 9.6 \pm 3.5$ $M_2 = 51.2 \pm 20.6$	6—1 080 (23) $M_1 = 220$ $M_2 = 920$		
Muscle spindle motor fiber			132—1 590 (7) M = 480		
sens. fiber			0.19—860 (14) M = 273		
Motor end plate (r. abdominis)				0.8—4.1 (5)	
Muscle fiber extrafusal					1.8—3.9 (4)
intrafusal					6—13 (3)

[1] The results were obtained by the diver method reported in the text using AThCh, ACh and Mipafox (5×10^{-4} M). The AThCh values are corrected according to note 2, table I. The variation range of the activity values is reported together with the mean value M, the mean values of different groups of cells or axons M_1 and M_2 and the number of experiments (in brackets). The expression "total activity" means activity per single cell body, nucleus etc. For comparison the values of AChE act. of single muscle fibers are reported. Total number of experiments = 215.

The ganglion cells investigated show a wide variation in ChE activity but cannot be classified into groups of cells of uniform type as obtained by means of the histochemical method.

The total ChE activity per single cell varies between 4.4 and 0.13 μl $\times$ 10^{-9}/hour in the frog and between 2.2 and 0.11 μl $\times$ 10^{-9}/hour in the rat. It can however be said that in the rat the cells exhibiting the highest activity (5—30 times higher than the rest) are far from numerous. Finally in both animals, in a certain number of cells, no activity could be demonstrated even if the measurement of the CO_2 evolution was prolonged in some cases to 8 hours. It could also be demonstrated that there was no correlation between the cell dimension as judged either by its computed diameter, surface area or volume, and the enzyme activity. The activity of the axon was well correlated with that of the cell body and the ratio between them varied between 1 and 10.

In later experiments with other substrates and inhibitors it could be established (GIACOBINI unpublished results) that in some sympathetic cells non spec. ChE was also present in lower amounts; however in most cells mainly AChE was acting.

Finally it could be seen that the activity of the sympathetic ganglion cells is lower than that of the anterior horn cells and of the same order as that of the spinal and parasympathetic ganglion cells.

The demonstration that the great majority of sympathetic cells contain in their cell bodies and axons a certain amount of enzyme is apparently contrary to what was assumed earlier (KOELLE 1950—54). The present finding is supported by the investigations of BROWN, MC LENNAN and PASCOE (1952) and MC LENNAN (1954) who by means of manometrical estimations of ChE in the superior cervical ganglion of the rat found that about 50 % of the activity was lost after section of the postganglionic nerves. This phenomenon was accompanied by the failure of ganglionic transmission while the acetylcholine output was still normal. These results have been recently confirmed histochemically (BROWN 1958).

The physiological interpretation of the results has been discussed in paper III (pages 241—242) and the hypothesis that the difference of AChE activity in the different cells is a sign of a different functional nature has been considered.

Pharmacological evidence for such a hypothesis has been produced by SHAW *et al.* (1945—51) (see paper III, page 242) and by

[1] The results were obtained by the diver method reported in the text using AThCh. ACh and Mipafox (5×10^{-5} M). The AThCh values are corrected according to note 2, table I. The variation range of the activity values is reported together with the mean value M, the mean values of different groups of cells or axons M_1 and M_2 and the number of experiments (in brackets). The expression "total activity" means activity per single cell body, nucleus etc. For comparison the values of AChE act. of single muscle fibers are reported. Total number of experiments = 215.

TRENDELENBURG (1956) who, after studying the modification of the ganglionic transmission following close intraarterial injections of different active substances, suggested that in the superior cervical ganglion of the cat there are present not only cells possessing ACh receptors but also cells having receptors for histamine, pilocarpine, 5-hydroxytryptamine and for sympathomimetic amines.

On the other hand, independent of this suggestion, there are now many facts which do not support the commonly accepted view that sympathetic ganglions contain exclusively cell bodies of noncholinergic nature, and which more directly support the biochemical findings reported above. The presence of cholinergic fibers in the sympathetic system (postganglionic fibers) has been suspected for many years and among the first observations of cholinergic sympathetic fibers are those of DALE and FELDBERG (1934) and those regarding the vasodilator nerves of the facial muscles of the cat (EULER and GADDUM 1931), the vasodilator fibers of the muscle of the hind legs of the dog (BÜLBRING and BURN 1935), the abdominal sympathetic fibers of the cat (ROSENBLUETH and CANNON 1935) and the vasodilator fibers to the hind legs of the cat (BÜLBRING and BURN, 1936).

More recently the sympathetic vasodilator innervation was reinvestigated by UVNÄS *et al.* (FOLKOW, HAEGER and UVNÄS 1948; FOLKOW, FROST, HAEGER and UVNÄS 1948—49; FOLKOW and UVNÄS 1950; UVNÄS 1954). These authors presented evidence for the fact that sympathetic vasodilator innervation both in the cat and in the dog is limited to the skeletal muscles and that the vasodilators are exclusively cholinergic.

Further, GOLLWITZ-MEIER and KRÜGER (1935) and FOLKOW, FROST, HAEGER and UVNÄS (1948) produced evidence that sympathetics carry cholinergic fibers to the heart in the cat and dog; and ERICI, FOLK and UVNÄS (1952) indicated the existence of cervical cholinergic vasodilator fibers to the tongue of the cat.

b) Parasympathetic ganglion cells.

In an earlier histochemical study (I) of the distribution of cholinesterases in individual parasympathetic cells isolated by microdissection from the ciliary ganglion of the rat it was seen that practically all the cells investigated contained high AChE activity in their cell bodies and axons. This result is in agreement with the previous histochemical investigations of KOELLE

in the cat (KOELLE 1951—55). Later, diver experiments (GIACOBINI unpublished results) on isolated cell bodies of the parasympathetic cells confirmed the results obtained by the histochemical method and gave more exact information on the enzyme activity of these neurones. Using acetylcholine as a substrate in the presence of Mipafox it could be established that AChE was acting either exclusively or predominantly in all the cells investigated.

The AChE activity of these cells varies remarkably little from one to another (between 1.2 and 4 μl $CO_2 \times 10^{-3}$/hour) and their activity per unit of volume was found to be the next highest, after the anterior horn cells, of all types of cells investigated (see Table II).

The findings are in agreement with the generally accepted theory stating that these neurones are of cholinergic type.

c) Spinal ganglion cells.

Earlier histochemical work on sections of spinal ganglions of different mammals (KOELLE 1951—55) demonstrated that primary sensory neurones exhibited uniformly little or no AChE activity at all.

The histochemical technique employed in this work (paper I) on isolated spinal ganglion cells of the frog and rat demonstrated with respect to AChE activity two types of cells: about 85—90 % of the total cell population exhibited only slight or no AChE activity after short incubation (15 min.) while about 10—15 % showed a high activity.

The crystals of copperthiocholine sulfate produced by the histochemical reaction were localized all over the surface of the cell body and the neurite.

In some cells non spec. ChE could be demonstrated by employing a selective substrate or a selective inhibitor.

No morphological difference could be observed between the cells which showed a high AChE activity and those with slight activity. In the ganglion the two types of cells were intermingled and did not show any special distribution. The results of the investigation of single spinal ganglion cells of the rat with the cartesian diver technique showed, in analogy with the above mentioned ones, that most of these cells have a measurable ChE activity and that a part of them also contains non spec. ChE. In a certain number however it was not possible to demonstrate

any ChE activity at all (c. f. Table I b, paper V). As Tables I a, I b and II demonstrate (paper V), the enzyme activity varies significantly (total activity between 3 and 0.06×10^{-3} μl CO_2/hour) from cell to cell, *e. g.* if the activity per unit of volume is considered this variation can be higher than one hundred times (see also Table II). From the same tables and from the graphic representations in Fig. 3 A and 4 (paper V) it can be seen that the enzyme activity could not be correlated with the dimensions of the cell (diameter, surface area or volume). The statistical computing of the data showed the presence of two different groups of cells having higher and lower activity. The activity values plotted against the diameter, the surface area and the volume were arranged along two separate curves of hyperbolic form (see paper V, Fig. 4). An analogous disposition was also evident for the neurites (see paper V, Fig. 5). The division into two separate groups of activities and the characteristic form of the curves reported in Fig. 4 and Fig. 5 closely resemble those of the anterior horn cells (paper IV) but differ from that of the sympathetic ganglion cells (paper III).

Finally, comparing the activity per unit of surface or volume of the cell body with the corresponding axon (see paper V, Table 3), the latter is found to be several times higher, the ratio varying between 8 and 131 (unit of volume).

With regard to the absolute activity per unit of volume, the spinal ganglion cells of the rat show the same variation range found in the sympathetic cells but about 10—20 times lower than that of the anterior horn cells (paper IV).

Loewi and Hellauer (1938) studying the ACh content of the ventral and dorsal roots of the cat suggested for the first time that ACh is not involved in the metabolism of primary sensory neurones.

It is generally agreed that dorsal root fibers are not cholinergic and that their central synapsis is not under the influence of ACh (Eccles 1948; Bremer 1953; Feldberg 1954). A biochemical support for this theory has been found in the low content of ACh, AChE and cholinacetylase of the dorsal root fibers (Loewi and Hellauer 1938; Chang *et al.* 1939; Mac Intosh 1941; Feldberg 1945; Feldberg and Mann 1946; Feldberg and Vogt 1948; Wolfgram 1954; Zetler and Schlosser 1955; Hebb and Silver 1956).

The ChE activity of whole spinal ganglions of the dog was determined by Nachmansohn (1939) who found an activity of 30—50 mg ACh/g tissue/hour, that is about 4—5 times lower than

that of the sympathetic ganglions and about 2—3 times lower than that of the grey matter of the spinal cord.

The ChE activity of whole posterior spinal roots of the dog, was calculated (BURGEN and CHIPMAN 1951) to be about 4—5 times lower than that of the anterior roots, but in cattle (WOLFGRAM 1954) about the same values have been reported for the dorsal and ventral roots.

The findings of this paper showing that a part of the sensory cells contain AChE could explain the small rates of ACh and ACh synthesis which have been found in the dorsal roots. (For a more complete discussion of this point compare paper V, discussion.)

The possibility that sensory cells of cholinergic type exist in spinal ganglions should therefore be borne in mind.

d) Anterior horn cells.

When ventral and lateral horn cells of the spinal cord of the rat, frog or cat, were incubated in a medium containing AThCh for about 5 minutes the appearance of the crystals of the histochemical reaction over and inside the cell body and axon demonstrated the presence of a very high ChE activity (paper I). About 30 minutes later the cytoplasm of these cells was completely filled with small crystals and at the same time they also appeared in the dendrites. Incubation with specific substrates and inhibitors demonstrated that the enzyme was AChE. An attempt to evaluate the degree of this activity in different parts of these neurones did not produce any valid results.

In a later investigation (paper IV) an attempt was made to find out if any difference in levels of ChE exists within a comparatively limited group of neurones of the spinal cord of the rat. For this purpose a group of neurones of the antero lateral part of the grey substance of the seventh segment was chosen; this group contains about 10—12 large cells whose neurites innervate extensor muscles of the fore limb of the rat.

In tissue sections (paper IV) stained with the thiocholine method, in spite of some diffusion of the histochemical end product the ChE activity was clearly demonstrable in the anterior horn cells and it could also be traced along the neurites issuing from these cells (paper IV, Fig. 3). The sections between C 6 and C 8 showed these heavily stained regions to be strictly localized in

the anterior and lateral horns; no cells in the posterior horns were stained histochemically.

The histochemical picture however could not definitely demonstrate any clear cut difference in the enzyme activity of the cells of the anterolateral region. The cartesian diver technique was therefore employed on single cells belonging to the same group of cells investigated histochemically. The results presented in paper IV (Table I) and in Table II (this paper) demonstrate that all the cells investigated contain ChE, that their activity varies between 0.71 and 6×10^{-3} $\mu l/CO_2$/hour, a much narrower variation than in the sympathetic and spinal ganglion cells, and that it is several times higher than in the latter. No direct correlation could be demonstrated between the ChE activity and either the cell diameter, cell surface area or cell volume. (See paper IV, Fig. 5.)

Two significantly different groups of cells could be demonstrated in this region, with respectively high and low ChE activity. If the computed diameter is taken as reference one group of cells shows approximately four times the activity of the other.

The high activity of cells pretreated with the selective inhibitor Mipafox indicated that AChE was predominantly operative.

In the same investigation, in vitro control experiments were carried out with rat brain and spinal cord homogenates and the selective inhibitor Mipafox. Experiments with partially purified preparations from the electric organ and human serum, were included for comparison. The results of previous investigations (ALDRIDGE 1953) that rat brain contains approximately 10 % non spec. ChE and 90 % AChE, were confirmed.

In later investigations (papers VI and VII), the ChE activity was determined in the cell body, cytoplasm, axon, dendrite, nucleus, nucleoplasm and nucleolus of anterior horn cells of the rat isolated from the same segment of spinal cord employed in the previous works, by means of a modified and more sensitive cartesian diver technique (paper VII). The results of these investigations have been summarized in Fig. 1, p. 25. They demonstrate that AChE is highly concentrated in the anterior horn cells and in their processes and that the enzyme is present in different concentrations in different parts.

The cell body exhibits the highest value of ChE activity, the axon and dendrites a somewhat smaller one, and the nucleus has an activity 10—100 times lower than the other parts (see Fig. 1).

In some experiments, when a determination could be made of the activity of the cytoplasm and of the axon of the same cell, the latter was found to be 1—3 times lower (to be published). The results concerning the intracellular localization of the enzyme are discussed in part II.

The earlier literature on this subject reveals that large concentrations of ChE were found in the grey matter of the spinal cord of the dog by NACHMANSOHN (1939) and BURGEN and CHIPMAN (1951) and by the latter in the ventral roots.

Acetylcholinesterase has also been previously demonstrated by means of a histochemical technique by KOELLE (1951—55) in the motoneurones of the cat and rat and according to this author the AChE activity appeared to be concentrated at the neuronal surface membrane. The same author reports however that in certain cells showing specially high activity the cytoplasm was also stained.

From the discussion in paper IV where a comparison is made of the results obtained from diver experiments and those obtained from the histochemical technique, it appears that even a semi-quantitative estimation of ChE activity by means of the latter technique has to be made very cautiously.

A detailed study of the functional significance of this group of neurones by means of degeneration experiments (GOERING 1928) and also the size and the localization of these cells demonstrate that they are motoneurones. Different theoretical possibilities can be taken into account (see paper IV, discussion, page 24—25) regarding the functional significance of the two groups of cells demonstrated by the diver technique, one of them seems however to be more likely, namely that they may correspond to the two groups of motoneurones: the slow discharging tonic ones innervating red muscle fibers and the rapidly discharging phasic ones innervating white muscle fibers according to the findings of GRANIT, HENATSCH and STEG (1956—57) in cat extensor and of ECCLES, ECCLES and LUNDBERG (1957) in different muscles of the cat.

From earlier work no certain evidence of an excitatory action of ACh on motoneurones had emerged (LOEWI and HELLAUER 1938, COOMBS, ECCLES and FATT 1955) but in a recent work (ECCLES, ECCLES and FATT 1956; ECCLES, FATT and KOKETSU 1954; CURTIS, ECCLES and ECCLES 1957; CURTIS and ECCLES 1958) evidence has been produced that the synapsis between the

motor axon collaterals and Renshaw cells is mediated by acetylcholine.

The possibility of a cholinergic central synapsis in the spinal cord is supported from a series of biochemical findings which enclude the high AChE activity of the neurite of the motoneurones demonstrated in this paper, and the very high content of ACh, choline acetylase and AChE in the grey matter of the spinal cord and anterior roots. These findings support the principle enunciated by DALE in 1935 according to which the chemical transmittors released from all the synaptic terminals of a neurone must be identical.

It seems therefore very reasonable to assume that the same chemical transmittor is released centrally by the terminals of the motor axon and perhaps of its cell body as is released peripherally by the terminals to the muscle, a site where the cholinergic nature of the transmission has been proved and investigated in great detail (for ref. see KATZ 1956).

e) Muscle spindles (afferent and efferent fibers, muscle fibers).

Morphological work (SHERRINGTON 1894; RUFFINI 1898; BARKER 1948) demonstrated that the mammalian muscle spindle is supplied with two types of sensory endings: the primary, localized in the central portion of the spindle and connected to the largest nerve fibers innervating the spindle, and the secondary which is also located in the central portion, but more towards the poles, and is connected to somewhat thinner fibers. The motor innervation of the muscle spindle consists of very thin nerve fibers from the ventral roots (γ efferents) terminating in the small motor end plates located in the polar regions of the spindle.

The most commonly used animal for study on the innervation mechanism of single spindles has been the frog, probably due to the fact that it offers particularly favourable conditions for the preparation of isolated muscle spindles.

In the investigation here reported, it was however found more convenient to choose the muscle spindles of the rectus abdominis of the rat. The spindles of this muscle are rather long, about 2—3 mm, and are formed by 3—5 thin muscle fibers (5—10 μ diam.).

The axons innervating the different parts of the spindles can be dissected separately and cut out after visualization of the spindle

both in stained and unstained preparations. In the former case a short segment of the muscle is incubated during 5—10 minutes according to the histochemical technique reported in paper I.

Short segments, measuring 100—400 μ of single thin (diam. 1.7—4.5 μ) fibers innervating the intrafusal end plates of the polar regions and similar segments of thick myelinated (diam. 6—10 μ) fibers connected to the sensory equatorial region were isolated and cut out by means of microdissection in fresh preparations immersed in a Ringer-bicarbonate solution. One or several segments of the same fiber were further introduced in different divers for the measurement of ChE activity, in the presence of various substrates and inhibitors (see Table I).

It could be demonstrated that the ChE present in the thin motor fibers innervating the intrafusal muscle fibers, is AChE (see Table II). BuCh was not split by these fibers while ACh and MeCh were split at high rates. The AChE activity varied between 1.32 and 15.9 μl $CO_2 \times 10^{-7}$/hour per unit volume. These values may be well correlated with those determined in the initial part of the neurite of the anterior horn cells (see Table II and part I d). In the thick myelinated fibers innervating the sensory equatorial zone of the spindle, both types of cholinesterase were found, but from the ratio of hydrolysis ACh/BuCh there could be calculated percentages of about 75 % AChE and about 25 % non spec. ChE. These findings raise the question as to whether these sensory fibers should be identified with the group of axons belonging to the spinal ganglion cells (see part I c) which were found to contain high concentrations of AChE together with low amounts of non spec. ChE.

In the rat, the intrafusal muscle fibers differ from the extrafusal in their coarser striation, smaller diameter and centrally placed nuclei. Further they do not degenerate as rapidly as the other muscle fibers when deprived of their motor innervation (SHERRINGTON 1894—95).

In a group of experiments (see Table II), the ChE activity was determined in about 100—250 μ long segments of single intrafusal muscle fibers. Both ChEs were found in these muscle fibers, with a prevalence (about 80 %) AChE upon non spec. ChE. The ChE activity per unit volume was very low, of the order of about 0.06 μl $CO_2 \times 10^{-7}$/hour, that is about one hundred times lower than in the motor or sensory fibers innervating the spindle, but still 3 times higher than in the extrafusal muscle fibers.

The possibility of a contamination of the muscle fibers with nervous material has however to be taken into account as it is very difficult to obtain by means of micromanipulation intrafusal muscle fiber preparations completely free from nervous structures.

Further evidence for the localization of the ChEs indicated by the diver experiments both in nerve and muscle fibers could be obtained by means of sections of the muscle stained by the histochemical method according to the technique reported in paper IV. (GIACOBINI and HOLMSTEDT to be published.)

In previous histochemical investigations (CÖERS 1954; MOHR 1954; CÖERS and DURAND 1956) the ChE activity of the muscle spindles was confined to the polar regions where it was localized into the motor end plates and into the muscle fibers. The equatorial zone containing the sensory (anulo-spiral) endings was considered to be totally devoid of the enzyme.

f) Muscle end plates.

In 13 experiments the ChE activity of single motor end plates of different size, dissected from the rectus abdominis of the rat was investigated (see Table II).

The end plates of this muscle offer some advantages compared with those of other striated muscles: they are placed in straight lines running at right angles to the direction of the muscle fibers and are at equal distances from the intermediary tendons. For this reason they can be identified and isolated relatively easily, even in unstained preparations. They are of particularly large dimensions and finally proved to be more convenient for dissection from the surrounding muscle tissue than those of other muscles (diaphragm, intercostal muscles).

In order to differentiate between different types of cholinesterases, three substrates (ACh, BuCh and MeCh) and two specific inhibitors (Mipafox and BW 284 c 51) were used. (HOLMSTEDT 1957 a.) The results of this investigation demonstrated that in the end plate of the rat two distinct types of cholinesterases are present, corresponding in their properties to those found in the nerve cells. The histochemical controls in tissue sections, confirmed these results (GIACOBINI and HOLMSTEDT to be published).

The AChE activity varies among the different end plates in-

vestigated between 0.8 and 4.1 μl $CO_2 \times 10^{-3}$/hour, while the non spec. activity varies between 1.5 and 2 μl $CO_2 \times 10^{-3}$/hour.

The complexity of the end plate morphology did not allow calculation of the concentration of the enzyme per unit of surface area or volume; however from an approximate calculation it is possible to establish a certain direct proportionality between the surface of the end plate measured in fresh intact preparations stained by the histochemical method and its ChE activity measured in the diver. This fact not only gives an explanation of the individual variations of the enzyme activity but also supports the findings of COUTEAUX and TAXI (1951—52) who with the help of the histochemical method of KOELLE and FRIEDENWALD (1949) localized the enzyme activity at the level of the subneural apparatus.

In connection with the present investigation it was found of interest to estimate the ChE activity of single thick motor fibers innervating the extrafusal muscle fibers of the same muscle. The enzyme of these nerve fibers was seen to split both ACh and MeCh but not BuCh. The AChE activity (10 experiments) varied between 1.30 and 23.4 μl $CO_2 \times 10^{-7}$/hour per unit volume and corresponds well (see Table II axon dist. part) to that determined in the initial part of the neurite of the anterior horn cells (part I d). Similarly the activity of these axons was not correlated to the axon volume or surface area.

Finally, some diver determinations were made on small segments (vol. 100—500 $\mu^3 \times 10^3$) of single muscle fibers dissected from the rectus abdominis (see Table II).

The fibers of this muscle were shown to contain both cholinesterases in very low amount. From the ratio of hydrolysis ACh/BuCh it can be calculated that they must contain approximately 80 % AChE and 20 % non spec. ChE. The ChE activity could be estimated to be of the order of about 0.018 μl $CO_2 \times 10^{-7}$/hour per unit volume, which is the lowest activity determined in the structures hitherto investigated. This activity is for example several hundred times lower than that found in the motor fibers innervating the muscle fibers and about 3 times lower than in the intrafusal fibers (see part I, e). The method employed, unfortunately, does not allow localization of the enzyme with more precision and leaves open the question as to whether the activity may be concentrated in the sarcolemmal structures or in the myofibrils themselves.

Table III.

ChE activity of single cell bodies and other portions of anterior horn-cells of rat. Surface and volume values were calculated as previously reported (see methods, paper VII).

Exp. no.	Cell part	Diam. (μ)	Length (μ)	Surf. (μ^2) ($\times 10^3$)	Vol. (μ^3) ($\times 10^6$)	ChE act./unit area μl CO_2/h. ($\times 10^{-7}$)	ChE act./unit volume μl CO_2/h. ($\times 10^{-9}$)	Total ChE act. μl CO_2/h. ($\times 10^{-9}$)
1	Cell body	28×16×8	—	0.74	1.8	17.9	736	+1 322[1]
2		31× 9×9	—	0.58	1.32	17.2	757	1 000
3		26×15×7	—	0.61	1.43	19.9	848	1 210
4		21×14×8	—	0.56	1.23	128.0	5 750	+7 200
5	Axon	1.3	40	0.16	0.053	0.675	208	+ 11
6		1.1	45	0.15	0.042	1.99	730	31
7		1.2	60	0.28	0.067	0.855	354	24
8		1.2	30	0.11	0.034	16.5	5 300	+ 180
9	Dendrite	1.6	35	0.17	0.070	1.41	341	+ 24
10		1.7	40	0.21	0.090	0.517	122	11
11		1.7	25	0.13	0.056	9.69	2 250	126
12		1.9	25	0.14	0.071	10.70	2 250	160
13	Nucleus	7×4×3	—	0.060	0.43	0.36	50	2.2
14		8×4×3	—	0.066	0.050	0.35	46	2.3
15		9×4×2	—	0.054	0.037	0.48	70	+ 2.6
16		11×5×3	—	0.94	0.086	0.54	59	+ 5.1
17		11×4×3	—	0.81	0.069	0.69	81	5.6
18		12×5×4	—	1.20	0.120	0.64	62	7.8
19		15×6×3	—	13.0	0.140	0.65	61	8.6
20	Nucleolus	3.8	—	0.045	0.029	0.007	1.1	0.03
21		4.2	—	—	—		no meas. activity	
22		3.9	—	—	—		no meas. activity	
23	Cytoplasm	—	—	—	0.01	—	480	+ 4.8
24		—	—	—	0.01	—	690	6.9
25		—	—	—	0.05	—	260	13.0
26		—	—	—	0.06	—	310	18.5
27	Nucleoplasm	—	—	—	0.01	—	34	0.34
28		—	—	—	0.01	—	41	+ 0.41
29		—	—	—	0.04	—	31	1.24
30		—	—	—	0.10	—	49	4.90
31		—	—	—	0.09	—	60	5.40

[1] The sign + precedes a figure expressing AChE activity alone.

In the pioneer studies of MARNAY and NACHMANSOHN (1938), COUTEAUX (1942) and COUTEAUX and NACHMANSOHN (1940—46) it was found that following section of the sciatic nerve of the guinea pig, the high concentration of ChE of the motor end plates of the gastrocnemius decreased only slightly and that the ChE could be localized at the level of the subneural apparatus of the end plates. On the basis of these results it was suggested that most of the enzyme was localized in the postsynaptic membrane, a muscular element which persisted after the degeneration of the innervating structures.

This view can be confirmed in the light of the results here reported and has also been confirmed in single end plate experiments of other muscles (BRZIN and ZAJICEK 1958), of the rat. It should however be enphasized that rather high concentrations of the enzyme (AChE) are also found in the presynaptic axons. This situation is the same as that which has been found in the sympathetic ganglions where the enzyme probably is present, at least in a certain number of cells, both pre- och postsynaptically (see part I a).

The significance of the localization of the enzyme in the end plate has to be related to the detailed study of the cholinergic mechanism at this level, obtained by the pharmacological and electrophysiological experiments of DEL CASTILLO, KATZ and FATT (1952—57) on single end plates.

The presence of the enzyme in the muscle fiber is of special interest since it has not been described before except in the myoblasts of the guinea pig (GEREBTZOFF 1957) and the muscle fiber of fishes (LUNDIN 1958), and because recent findings indicate that the activity of cholinesterase is a prerequisite for the working of the sodium pump in the muscle (VAN DER KLOOT 1956). VAN DER KLOOT demonstrated that the anti-cholinesterases which can penetrate into the muscle fiber and inhibit the intracellular enzyme are also capable of blocking the sodium pump. The experiments to which references have been made, have been performed on frog muscle. Further, the AChE of the muscle fibers, even if it represents a small fraction of the total ChE of the muscle, may, at least partially, be responsible for the enzyme activity which is left after denervation (MARTINI and TORDA 1937; MARNAY and NACHMANSOHN 1937; LEIBSON 1939; COUTEAUX and NACHMANSOHN 1940; MENG 1940; COUTEAUX 1942; STOERK and MORPETH 1944; BROOKS and MYERS 1952; BROOKS and CHIPMAN 1952).

Non spec. ChE was demonstrated in previous histochemical investigations in the smooth muscle of the intestinal wall of the cat (KOELLE 1950, 1951), in the cardiac muscle fiber of the dog and cat (HOLMES 1957), and in the smooth muscle fiber of the pulmonary trunk and aorta (HOLMES 1957).

It should finally be remembered that a third localization of AChE in the muscle was demonstrated in the "appareil musculotendineux" (COUTEAUX 1953; GEREBTZOFF 1954; CÖERS and DURAND 1957; GIACOBINI and HOLMSTEDT to be published).

PART II.

Intracellular Distribution of AChE in the Nerve Cell.

A technique for the determination of ChE activity in $\mu\mu$l of cyto- and nucleoplasm (papers VI and VII) was developed in the attempt to obtain a more exact intracellular localization of the enzyme.

The large anterior horn cells of the spinal cord of the rat were found to be most suitable for a detailed study of the distribution of ChE in the neurone.

From earlier investigations (papers I and IV) it is known that these cells contain a high amount of the enzyme.

The results of this study have been preliminarily reported in paper VI and in detail in VII. The technique employed, a modification of the diver technique, is described in paper VII.

From the results it has been possible to get an idea of the distribution of the enzyme in different parts of a neurone. From Fig. 1 where the results are schematically reported and summarized, it becomes evident that the enzyme is not strictly localized to a part of the cell but is present in the different concentrations in different parts (see also Table III). This figure shows the ChE activity curves per unit of volume (μ^3) of the cell body, cytoplasm, axon, dendrite, nucleus, nucleoplasm, and nucleolus of anterior horn cells of the spinal cord of the rat. The curves for the cell body, the axon and the dendrite refer to the same cell, the values of which have been taken from Table III (exp. no. 1, 5 and 9), and the other from the mean ChE values, calculated from Table III.

Fig. 1 demonstrates that the cell body, the cytoplasm and the

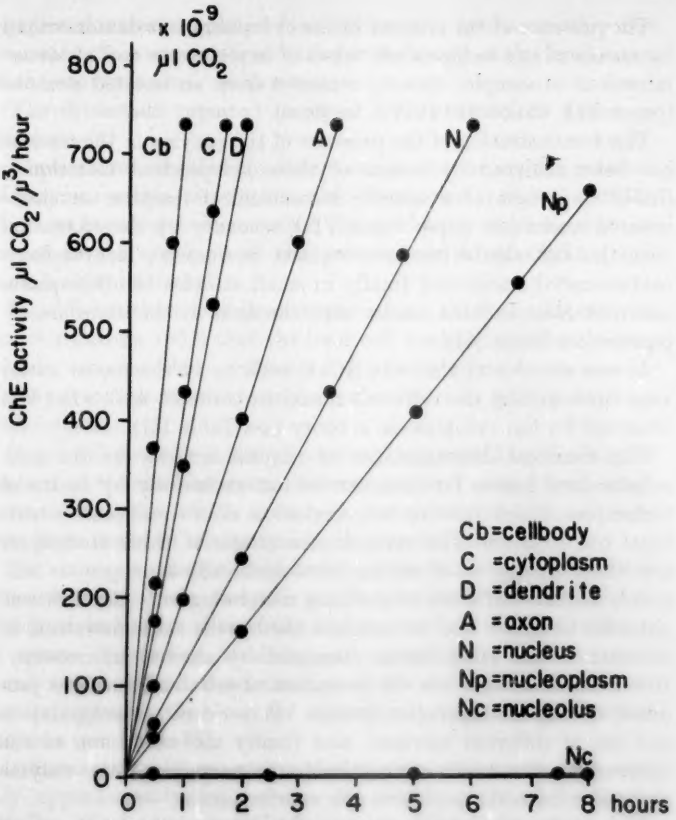

Fig. 1. ChE activity curves per unit of volume of different parts of an anterior horn cell of the rat. (See text.)

dendrite exhibit the highest ChE activity, followed by the axon, and finally by the nucleus having an activity 10—100 times lower than that of the cytoplasm. The nucleolus shows no activity at all. From Fig. 1 and Table III it can also be deduced that the ChE activity of the nucleus was higher when it was measured in the nucleus in toto than when measured in small samples of nucleoplasm. This fact is probably due to contamination of the nucleus with highly active cytoplasmic material or to the location of ChE on the nuclear membrane.

The presence of the enzyme in the cytoplasm was demonstrated by means of two independent types of experiments and by determinations in samples directly removed from an isolated neurone (paper VII; GIACOBINI 1959 c, in press).

The demonstration of the presence of the enzyme in the nucleus has been achieved by means of three independent techniques: firstly by means of a specific histochemical reaction on single isolated nuclei (see paper I and VII), secondly by determination with the cartesian diver in cytoplasm free nuclei, in cytoplasm contaminated nuclei and finally in small samples of nucleoplasm removed from isolated nuclei with the help of the diver micropipette (see Table III).

It was also found that the AChE activity of the nuclei varies very little among the different nuclei in contrast with what was observed for the cytoplasmic activity (see Table III).

The chemical determination of enzyme activity at the subcellular level has so far been carried out exclusively by means of techniques which involve fragmentation of the cell and centrifugal fractionation. The main disadvantages of these techniques are: the difficulty of obtaining histochemically homogenous material, and the difficulty of defining morphologically the different particles obtained and to correlate them with those described in sections of the same tissue examined by electron microscopy. Other disadvantages are the alteration of cellular structures produced during the separation process by mechanical manipulation and use of different solvents, and finally the extraction of the enzymes by the media with subsequent passage of the enzyme molecules from the primitive site to other sites.

The study of the ChE activity of isolated nuclei by means of the diver technique may be of special interest due to the fact that at present it is not yet possible to isolate an intact nuclear fraction by means of fractionation techniques in a pure enough state to allow a reliable determination of enzyme activities to be made.

ALLFREY, MIRSKY and STERN (1955) in a recent paper on the chemistry of the nucleus therefore stressed the necessity of being very cautious in interpreting results of experiments on the localization of enzymes in nuclei by means of fractionation methods.

The critical comparison of the data obtained with the modified diver technique in small samples of material removed from single cells and nuclei with the data obtained by determinations on big

samples of material isolated from the brain in toto seems therefore to constitute a more critical basis for the study of the localization of the enzyme in cell components.

The discussion reported in paper VII is therefore based on this concept.

There exists considerable disagreement concerning the presence of enzymes within the nucleus (see HOGEBOOM *et al.* 1953, 1955). There is however evidence that the nucleus is in some way involved in the synthesis of the proteins and of the RNA (ribonucleic acid) of the cell.

In addition there is indirect experimental evidence (HOGEBOOM and SCHNEIDER 1952) that the liver cell nucleus contains a water-soluble enzyme that synthetizes diphosphopyridine nucleotide (DPN) from nicotinamide mononucleotide and ATP (adenosine triphosphate).

In general it can be said that concrete data regarding the enzyme content of the nucleus are rather meager, and with a few exceptions the enzymes which have been demonstrated in the nucleus have been found in relatively low concentrations.

The only quantitative data found in the literature on the ChE activity of nuclei of nerve cells are those of RICHTER and HULLIN (1951) on isolated nuclei of the human cerebral cortex obtained, by means of grinding, straining and centrifuging. These authors found a considerable concentration of the enzyme in the nuclear fraction (greater than in the cytoplasm) together with other enzymes (see Table IV).

It is necessary to remember that the isolated nuclei represent only approximately 5 % of the dry weight of the whole tissue, therefore the largest part of the enzyme will still be retained by the cytoplasm. It should be further pointed out that of all fractions obtained from sucrose homogenates, the nuclear one is the most contaminated and it contains in addition to nuclei, cytoplasmic particles, unbroken cells, erythrocytes, cell membranes, microsomal and mitochondrial material. In addition it has been demonstrated by electron microscopy that alterations to the nuclear structure occur, due to the separation procedure (NOVIKOFF 1956). On the contrary the results obtained with the diver pipette technique exclude any possibility of contamination with cytoplasmic material and also avoid at least partially a crude manipulation of the material.

Among the first works on the intracellular localization of ChE

Table IV.

The intracellular localization of the ACh-ChAc-ChE system in the nervous tissue.[1]

	Species	Cell membrane	Nucleus	Cytoplasm			References
				Mitochondria	Microsomes	Supernatant	
ACh	rat			+++			Brodkin and Elliott, 1953
	dog			+++			Stone, 1955
	frog			+++			Loewi, 1956
	homo					+++	Hagen, 1956
	rabbit				+++ (synaptic vesicles)		Whittaker and Hebb, 1958
ChAc	rabbit	+	+	+++ (50—70 %)	+	++ (25 %)	Hebb and Smallman, 1956
	homo					+++	Hagen, 1956
	rabbit				+++ (synaptic vesicles)		Whittaker and Hebb, 1958
AChE	squid (axon)	+++					Boell and Nachmansohn, 1940
	homo		+++		++ (cytoplasm)		Richter and Hullin, 1951
	rabbit			+	+++		Nathan and Aprison, 1955
	insects				+++		Smallman and Wolfe, 1956
	rat			++	+++	++	Whittaker, 1958
	rat			+	+++	+	Toschi, 1958
	rat		+		++ (cytoplasm)		Giacobini, 1957

[1] High amount = +++; moderate amount = ++; low amount = +.

in the nervous system is that of BOELL and NACHMANSOHN (1940) which is also one of the first applications of the diver technique for an enzyme determination. These authors showed that in the giant axon of the squid, all the ChE is localized in the sheath, the axoplasm being practically free of enzyme (see Table IV).

More recently some investigations have been made by means of fractionation techniques on homogenates of brain: the investigation of RICHTER and HULLIN (1951) has already been reported.

The distribution of ChE in different fractions of sucrose homogenates of rabbit brain was studied by NATHAN and APRISON in 1955. They found that the fraction containing the large cytoplasmic particles and the mitochondria contained 70 % of the cholinesterase activity and that the highest cholinesterase activity was found in the microsomes. The soluble tissue fraction showed only 8 % of the total enzyme activity (see Table IV). The fact that over 90 % of the brain cell cholinesterase is found in the cytoplasmic particulates supports the results obtained with the diver technique reported in paper VII and Table III.

More recently TOSCHI (1959) could confirm the results of NATHAN and APRISON by using homogenates of rat brain and finding the largest part of the ChE activity in the particulate cytoplasmic fractions (mitochondria and microsomes) and only a small amount in the nuclear fraction. The highest specific activity was found by this author in the microsomal fraction. According to TOSCHI, a firm association exists between AChE and the 40—60 Å thick membrane structures of this fraction. These membranes, according to electron microscopy (HANZON and TOSCHI 1959) are considered to originate from the endoplasmic reticulum of neurones (PALADE 1954; PORTER 1953; SJÖSTRAND 1953; PALAY and PALADE 1955). It should be pointed out that this fraction should contain the "synaptic vesicles" which have been described in thin sections of brain by PALAY and PALADE (1955).

Similarly it was found by SMALLMAN and WOLFE (1956), that in homogenates of insect nervous tissue most of the enzyme is contained in the larger cell fragments although a certain part is localized in the microsome fraction.

Another fact to be taken into consideration is that in all types of cells investigated with the diver technique no direct correlation has been found between the activity of the cell body or axon and

their dimensions judged either as the diameter, the surface area or the volume.

The fact that no correlation exists between enzyme activity and the surface area of the cell body or of the axon is hardly in agreement with the view that the enzyme should be specifically localized at the surface of the cell body and the axon, or in other words in the membrane of the cell.

The results of the authors employing homogenates of nervous tissue and those obtained by the diver technique indicate that the enzyme is localized in the cytoplasm and in the processes of the nerve cell where it is mostly bound to the microsomes and that a small part of it is also present in the nucleus (anterior horn cells).

In order to clarify this interpretation it should be mentioned that it has been found (PALADE and SIEKEWITZ 1956) that microsomes are morphologically identical with the vesicular and tubular elements of the endoplasmic reticulum of intact cells.

This localization does not eliminate the possibility of the presence of the enzyme in lower amounts in the membrane of the cell, taking into account the modern concept of the cell membrane as recently described by electron microscopy (see PORTER 1953—54; PALADE 1954—55; PALADE and PORTER 1954). According to this concept the outer cell membrane extends into the inside of the cell forming a network of cavities separated from the rest of the cytoplasm, the membrane being partially constituted by microsomes.

In this connection it is also worth considering that there exist indications of a spatial separation within the cell of the two enzymes, AChE and ChAc (cholinacetylase), involved in the metabolism of ACh. It has been found by HEBB and SMALLMAN (1956) (rabbit brain) that 52—69 % of the total ChAc activity is concentrated in the mitochondrial fraction (see also HEBB and WHITTAKER 1958) (see Table IV).

There are also facts suggesting that the ChAc (HEBB and WAITES 1956) of the nerve trunk is derived from the cell body of the neurones, in analogy with AChE (SAWYER 1946).

All indications regarding the localization of ChAc in the cell body of the nerve cell are however of an indirect nature while for AChE this can be directly demonstrated with the cartesian diver technique.

Regarding the intracellular localization of the third component of the system, the ACh, it may be suggested on the basis of recent study (BODIAN 1942; BRODKIN and ELLIOT 1953; STONE 1955; LOEWI 1956)

that it could be bound to tissue structures such as mitochondria. The identification of mitochondria or of other ACh-bearing particles has also been attempted at synaptic and axonic level and according to a suggestion of ROBERTSON (1953—54) these could be identified with the small "synaptic vesicles" present in the axoplasm of nerve terminals or at nerve terminals (see also DE ROBERTIS and BENNET 1954; PALADE 1954 and DE ROBERTIS 1955—56). WHITTAKER and HEBB (1958) have recently brought further evidence for a binding of ACh to such "synaptic vesicles". In summary it can be said that we should deal with a cytoplasmic enzyme system, two components of which are probably both localized in the mitochondria or in the synaptic particles or in special particles or organelles (ACh and ChAc) and the third (AchE) in the microsomes. The physiological value and significance of such a disposition can at present only be suggested. In order to illustrate this distribution a schematic report is given in Table IV of the results of recent investigations concerning the intracellular localization of the components of the ACh-ChAc-ChE system in the nervous system of different species. All the data reported in this table were obtained by means of fractionation methods, except two of them which were obtained by means of the cartesian diver technique (BOELL and NACHMANSOHN 1940; GIACOBINI 1957).

The distribution of AChE which has been found in the different types of neurones studied in this investigation and the intracellular localization of the enzyme, when integrated with the present knowledge of the distribution and function of ACh and ChAc, are compatible with the theory of an implication of ACh in the chemical transmission mechanism. On the other hand the presence of the enzyme in a number of neurones in which the function of ACh has not yet been proved is at present difficult to interpret but suggests that the cell population of certain ganglions of the nervous system is not so homogeneous from a pharmacological point of view as believed previously. Equally obscure is the significance of the absence of the enzyme in certain cells (sympathetic ganglions, spinal ganglions).

On the other hand the significance of the different enzyme levels demonstrated in cells of different or of the same origin seems in need of elucidation. This will probably be obtained by means of pharmacological and electrophysiological experiments.

Finally, the essential role of AChE in the conduction mechanism of the nerve impulse has been stressed, principally by NACHMAN-

SOHN and his co-workers. This theory has been mainly based on experiments in which nerve fibers (crab nerve) were exposed to the action of ChE inhibitor substances (DFP and eserine) in the attempt to demonstrate that conduction was not possible in the complete absence of the enzyme (WILSON and COHEN 1953).

According to the calculations of NACHMANSOHN on the quantity of enzyme required for mantaining conduction, a splitting activity of 400—500 μg of ACh/g nerve/hour would be adequate to account for one or two million impulses during one second.

From the results of the investigations reported in Part I it may be deduced that the splitting capacity found in all types of cells investigated is more than several thousand times higher than the value that is completely adequate in this regard.

The significance of such a high concentration of AChE in neurones, some of which were previously considered to be non cholinergic, as well as the difference of activity between one neurone and another belonging to the same ganglion or region cannot be understood at present but has to be taken into account when the pharmacological and electrical properties of these neurones are investigated. The possibility of a supplementary function of the enzyme, different from the implication in the transmission mechanism, has also to be kept in mind.

PART III.

General Considerations.

a) The AChE activity of different types of nerve cells.

Table II shows the results of 215 determinations of AChE activity in individual nerve cells or parts of cells, muscle end plates and muscle spindles of the rat. The activity per single cell, nucleus, end plate etc. (total activity) is reported together with the activity per unit of volume.

It can be seen that the activity per cell or per unit of volume varies widely not only from one type of cell to another but also among cells of the same type. In some cases (anterior horn cells) the AChE activity may vary in the same group of cells up to 100 times. The anterior horn cells were found to exhibit the highest AChE activity of all the cells investigated. Their activity per unit of volume was found to be 3—20 times higher than in the para-

sympathetic ganglion cells, 10—20 times higher than in the sympathetic cells and 30—70 times higher than in the spinal ganglion cells.

With the help of a selective inhibitor (Mipafox) it could be demonstrated that the enzyme present in the anterior horn cells and in the parasympathetic cells investigated is AChE while in some of the spinal and sympathetic ganglion cells low amounts of non spec. ChE were found.

It should also be pointed out that in the spinal and sympathetic ganglions a certain number of cells did not show any measurable activity while in the anterior horns and in the parasympathetic ganglions all the cells investigated showed some degree of AChE activity.

The distribution pattern found in the cell body and in the axon of the various cell types varies from one cell type to another, for example in the anterior horn cells it differs from that found in the spinal ganglion cells (V).

In the former, the concentration of the enzyme in the axon is the same or lower, while in the latter the axon shows an activity 10—100 times higher, than in the cell body. In the sympathetic cells (see Table II) the activity of the cell body compared with that of its axon is found to be the same or 2—9 times higher.

An hypothesis which could perhaps explain such a difference in the intracellular distribution of the enzyme in cells or various types is that the enzyme is present in higher concentration in the cell bodies which form part of a reflex arch (sympathetic ganglion cells, anterior horn cells), but in lower amount in those which do not take direct part in it (*e. g.* spinal ganglion cells). In the latter case a migration process of the enzyme from the body to the axon could be responsible for the difference.

The synthesis of the enzyme in the cell body and its transport down to the axon is supported from other observations: Sawyer (1946) found that on cutting the sciatic nerve the AChE content in the peripheral nerve stump fell to about 40 % but in the central end it rose to about 300 %. Similar observations have been reported by Feldberg (1945), Banister and Screase (1950) and Hebb and Waites (1956) for choline acetylase. Lewis and Huges (1958) produced evidence for a migration of the enzyme from the motor cells down to the myoneural junction of Xenopus levis.

Such an hypothesis is also supported by evidence that there is

a slow flow of material along the axon as proved by the experiments of LUBINSKA (1954) in isolated axons and by WEISS and HISCOE (1948) in regenerating nerve fibers.

In this connection it should also be remembered that morphological investigations (BARR 1939; WYCKOFF and YOUNG 1956; ARMSTRONG and YOUNG 1957; DAVID 1957) suggest that the greater part of a post-synaptic cell surface is covered by "boutons terminaux". These nerve endings probably contain AChE-bearing structures as demonstrated histochemically by SZENTAGOTHAI (1954—57) in the ciliary ganglion cells and may, at least partially, be present in the single cell preparations used as material for diver determinations and therefore contribute to the high AChE activity of the post-synaptic cell bodies.

It should finally be stressed that the axon of the ChE bearing cells of spinal ganglions shows an activity of the same order as that found in the anterior horn cells. This fact may be of special interest since it has been demonstrated that some of these fibers are those which take part in an AChE containing reflex arch (see part III, b).

For comparison, Table II also includes the values of the AChE activity of single intra- and extrafusal muscle fibers isolated from the rectus abdominis of rat. It can be seen that the lowest concentration ever found in any of the structures investigated is that present in the muscle fibers: *e. g.* the activity of the extrafusal fibers was found to be several hundred times lower than that of the motor fibers innervating them (see part I, f). In both types of muscle fibers, both cholinesterases could be demonstrated, with a prevalence of AChE over non spec. ChE.

Table II shows finally the AChE activity values of single motor end plates isolated from the same muscle. Only the total activity of single end plates is reported; however, it can be seen that this varies widely in the material investigated from one end plate to another. Also in the motor end plate of the rat moderate amounts of non spec. ChE could be demonstrated both histochemically and with the diver technique, by means of specific inhibitors (part I, f).

b) Distribution of AChE in a synaptic reflex arch.

In recent years the attention of many neurophysiologists has been focused on the monosynaptic reflex (LORENTE DE NÓ 1938; ECCLES 1946; LLOYD 1946; BERNHARD 1947—53; BROOKS and

Eccles 1947; Bremer and Bonnet 1949; Brock, Coomb and Eccles 1951; Brooks 1953) because it represents the simplest situation where the basic phenomena of nervous activity may be studied in detail, especially from the electrophysiological point of view. The biochemical and in particular, the enzymological aspects of such phenomena as conduction and transmission along a whole synaptic reflex arch are less well known, due to the difficulty of finding methods sensitive enough to allow chemical analysis of the single components of the arch itself.

Studies with quantitative micromethods such as the cartesian diver in conjunction with microdissection offer the possibility of approaching the problem of the distribution pattern and the physiological significance of different enzymatic systems in various nervous pathways. (Giacobini 1958 b.)

The results summarized in part I, c, d, e, f, of this paper show that it is possible to obtain an idea of the distribution of the cholinesterases along a whole reflex arch.

The components of the arch which can be examined are: the anterior horn cells, the afferent and efferent fibers to the muscle spindle and the spinal ganglion cells.

The anterior horn cells show high amounts of AChE in their cell bodies and neurites (see part I, d and paper IV). If the axons of these cells are analyzed peripherally, near their endings in the muscle, the same concentration of the enzyme is still found (see part I, e and f). This is true both for the large axons (α efferents) connected to the muscle end plates (part I, f) and for the thin fibers (γ efferents) innervating the small intrafusal end plates (part I, e). The situation is different in the large afferents (see part I, e) from the muscle spindles where both enzymes are present. AChE is however largely predominant over non spec. ChE.

If we finally consider the ChE activity of the spinal ganglion cells (part I, c) we find that a relatively large group of cells contains AChE together with a low amount of non spec. ChE. It seems reasonable to assume that the large afferent fibers from the muscle spindles are connected with these AChE containing ganglion cells.

In other words the cartesian diver results indicate that we are dealing with a whole synaptic AChE containing reflex arch.

It should also be remembered that according to the results obtained with the histochemical and the cartesian diver methods low amounts of this enzyme (AChE) are probably present in the

intrafusal muscle fiber. In addition, non spec. ChE is present in the extrafusal and intrafusal muscle fibers and in the afferent fibers.

The concentration of the enzyme varies in the different constituents of the reflex arch, the efferent part shows the highest concentration, the afferent one somewhat lower and finally in the muscle fibers it is about one hundred times lower. The physiological significance of such a gradient of the enzyme concentration in the different constituents of a reflex arch is not understood at present. FELDBERG and VOGT (1948) studying the distribution of cholinacetylase in the nervous system of the dog put foward a theory according to which there exists in the central nervous system a succession of alternating non-cholinergic and cholinergic neurones. Evidence for such a succession was found in the voluntary motor pathway, in the optic way and in other sensory pathways.

If we consider in the light of the present investigation the distribution of the AChE in four types of synapses, in the parasympathetic ganglions, in the sympathetic ganglions, in the spinal cord, and in the myoneural junction, we see that the theory enunciated above does not apply. Namely in all four synapses, on the basis of histochemical and diver experiments, it was possible to demonstrate the presence of the enzyme both in the pre- and in the post-synaptic neurones.

These results are in agreement with the findings of SZENTAGOTHAI (1954—57) who by means of the histochemical thiocholine method was able to demonstrate AChE activity both in the pre- and post-synaptical structures of the ciliary ganglion cells of birds and reptiles.

It should however be kept in mind that hitherto only in two of the four types of synapses, that is in the sympathetic ganglions and in the myoneural junction, a cholinergic transmission mechanism has been demonstrated and finally that the demonstration of AChE alone cannot be accepted as an absolute proof for the presence of a cholinergic type of transmission.

On the other hand, in view of the lines of evidence presented in this chapter and their possible consequences it is suggested that the terms cholinergic and non-cholinergic neurones, and the assumption that AChE is present exclusively in the former, have to be viewed critically.

c) ChE in the glial and vascular tissue of the nervous system.

The question as to whether the cholinesterases are present or not in the glial and vascular tissue is of primary importance for the study of the localization of these enzymes in the nervous tissue. The presence of such enzymes in non-nervous tissue of the brain cord and ganglions which is intermingled with the nervous elements may be a serious source of error when estimating the ChE activity of these structures in toto.

KOELLE (1951) using a histochemical method, reported the presence of non spec. ChE in the glial cells of the spinal and sympathetic ganglions and in the Schwan cells of myelinated nerves. The same author (1951—52) also suggested that non spec. ChE is present both in the fibrous astrocytes and in the vascular endothelium of the spinal cord of the cat. The findings of KOELLE on the vascular tissue were later confirmed by the biochemical investigation of THOMPSON and THICKNER (1953) who showed that the arteries dissected from the human brain contain active cholinesterase which is predominantly non spec. ChE.

HEBB, SILVER, SWAN and WALSH (1953) found non spec. ChE in the glial cells and septa of the rabbit's optic nerve.

In 1955, KOELLE confirmed his earlier findings in cat tissue in the central nervous system of the rat, where he was able to show heavy staining of glial cells and capillaries. A further support to these results has come from studies on the ChE content of a variety of different brain tumours.

The first who measured the cholinesterase activity in pathological nervous tissue were YOUNGSTROM, WOODHALL and GRAVES (1941). They found that tumours of the astrocytic series showed considerably more activity than neuromas, meningiomas, or medulloblastomas. In 1953, BÜLBRING, PHILPOT and BOSANQUET found that tumours of the astrocytic series and ependymoma gave the highest values of non spec. ChE. CAVANAGH, THOMPSON and WEBSTER (1954) studied the ChE activity of 22 cerebral tumours. These authors found that the astrocytic tumours showed very high levels of activity as compared with normal brain and that meningiomas and juvenile medulloblastoma showed only low activity.

Further support to the extranervous localization of the non spec. ChE is coming from degeneration experiments of SAWYER

(1946) and of CAVANAGH, THOMPSON and WEBSTER (1954) who demonstrated that non spec. ChE was practically non affected after complete disintegration of the nerve fibers. From these experiments it was concluded that this enzyme is wholly a sheath component, situated probably in the Schwann cells.

Finally it should be remembered the investigation of LUMSDEN (1957) who studied the alterations of the ChEs in certain types of plaques in multiple sclerosis. The results obtained by this author suggest that non spec. ChE has a dual source from oligodendrocytes and from microglial hystiocytes.

Using the histochemical method on single cells (paper I) it was seen that the capsular glial cells of the sympathetic and spinal ganglions of rat showed a strong positive reaction for non spec. ChE. The same enzyme could also be demonstrated in single neuroglial cells (protoplasmic astrocytes) isolated from the spinal cord of the rat. The results could later be confirmed by experiments with the cartesian diver technique on the same material (unpub. res.).

In conclusion, the observations here reported, indicate that the glial cells and the vascular tissue in the central and peripheral nervous system contain non spec. ChE.

General summary.

The object of the present survey is to summarize and discuss the experimental results obtained by the author in a series of investigations on the distribution and localization of cholinesterases in nerve cells of different type.

The results of the single investigations are reported in detail in the 7 papers listed on page 3 and quoted in the course of the test with corresponding roman figures.

1. In the introduction is reported the definition and nomenclature of cholinesterases together with the purpose of the investigation.

2. In part I are summarized the results of the original investigations on the distribution of ChE in nerve cells and nerve structures isolated by means of microdissection. The animal which has been mainly employed is the rat.

From the cholinesterase analysis of single medium sized and small nerve cells and 100—250 μ long segments of their axons it could be demonstrated that AChE is highly concentrated in

the neurones and their processes and present in various concentrations in the various parts. With the help of specific substrates (ACh, AThCh, BuCh, BuThCh and MeCh) and inhibitors (Mipafox and BW 284 c 51) it was shown that AChE is present in different amounts in some of the cells of sympathetic and spinal ganglions and in all the anterior horn cells and the parasympathetic ganglion cells investigated.

Non spec. ChE is found in low amounts in some spinal and sympathetic ganglion cells. Finally a certain number of cells in the spinal and sympathetic ganglions does not show any activity at all. In all types of cells investigated no direct correlation was found between the AChE activity of the cell body or axon and their dimensions judged either as the diameter, the surface area or the volume.

Both AChE and non spec. ChE are found in the muscle end plates of the rat. Low amounts of AChE and non spec. ChE were also detected in the extrafusal and intrafusal muscle fibers of the rectus abdominis of the rat. These results are discussed in the light of the previous literature.

3. In part II are reported the results on the intracellular localization of AChE studied with the help of the modified diver technique, reported in paper VII.

The determination of this enzyme was performed in $\mu\mu$l samples of cytoplasm and nucleoplasm, single nuclei and nucleoli and small segments of axons and dendrites isolated from single anterior horn cells.

The cell body (cytoplasm) exhibits the highest values of AChE activity, the axon and the dendrites slightly lower activity and the nucleus 10—100 times lower than the other parts. The nucleolus does not show any measurable activity. The indications for a cytoplasmic localization of AChE obtained by the cartesian diver technique are discussed in the light of recent findings on the intracellular localization of this enzyme in the nervous tissue obtained by fractionation techniques.

4. Part III deals with some general considerations on the distribution of cholinesterases in different types of nerve cells.

A difference is found in the intracellular distribution of AChE in cells from different sites: for example, in the rat, the distribution pattern in the cell body and in the axon of the anterior horn cells and in the sympathetic ganglion cells differs from that found in the spinal ganglion cells.

The presence of AChE at the level of four different synapses and in a whole synaptic reflex arch is commented.

Finally, the experimental results obtained with the histochemical and the cartesian diver method confirm the previous assumption that the glial cells contain non spec. ChE.

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